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Evaporative Light Scattering Detection of Pyrrolizidine Alkaloids

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A reverse-phase high-performance liquid chromatography method utilizing evaporative light scattering detection (ELSD) has been developed for the simultaneous detection of hepatotoxic pyrrolizidine alkaloids with and without chromophores, namely, riddelliine, riddelliine *N*-oxide, senecionine, senecionine *N*-oxide, seneciphylline, retrorsine, integerrimine, lasiocarpine and heliotrine. Pyrrolizidine alkaloids were detected in five plant extracts (*Senecio spartioides*, *S. douglasii var. longilobus*, *S. jacobaea*, *S. intergerrimus var. exaltatus* and *Symphytum officinale*). The detection of heliotrine (which does not contain a chromophore) was much improved by ELSD compared with photodiode array detection.

Keywords: HPLC; evaporative light scattering detector; pyrrolizidine alkaloids; *Senecio*; *Symphytum*.

INTRODUCTION

Over 6000 plants within the families Asteraceae, Boraginaceae, Compositae and Leguminosae produce more than 350 pyrrolizidine alkaloids in the form of free bases and corresponding *N*-oxides (Stegelmeier *et al.*, 1999). Many of the pyrrolizidine alkaloids have been shown to be hepatotoxic and carcinogenic in humans and animals (Molyneux *et al.*, 1991; Fu *et al.*, 2001). Up until 2001, manufacturers of dietary supplements were selling products containing comfrey (*Symphytum officinale*) which contained toxic pyrrolizidine alkaloids. Once toxicity was determined, the US Food and Drug Administration requested manufactures to remove all dietary supplements containing comfrey from the market (Food and Drug Administration, 2001). Some products containing comfrey are still commercialised, but only as skin care creams and are not to be used where a break in the skin is evident. The pyrrolizidine alkaloids also have a negative effect on livestock in the western USA. Plants of the genera *Crotalaria*, *Amsinckia* and *Senecio* grow wild in the grazing lands of cattle, horses and sheep (Molyneux *et al.*, 1991). Continued ingestion of the plant material, which contains toxic pyrrolizidine alkaloids, will lead to death. Unfortunately, the toxic effects cannot be overcome since the animals do not show symptoms until it is too late. To further the problem, contamination of human food sources by pyrrolizidine alkaloid residues has been found in meat, wheat, milk and honey (Edgar *et al.*, 2002). Methods for the analysis and detection of the pyrrolizidine alkaloids are therefore essential in preventing ingestion of any plants or products containing these toxic alkaloids.

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Many methods have been reported for the separation and identification of pyrrolizidine alkaloids, i.e. TLC (Molyneux and Roitman, 1980), capillary GC (Witte *et al.*, 1993), supercritical fluid chromatography (Holzer *et al.*, 1987) and HPLC (Brown *et al.*, 1994). The most common method of analysis is HPLC with UV detection, but his method is limited in that those pyrrolizidine alkaloids not having a chromophore in their structure are not detected. This paper reports an HPLC method utilizing an evaporative light scattering detector (ELSD). Nine pyrrolizidine alkaloids, riddelliine (**1**), riddelliine *N*-oxide (**2**), senecionine (**3**), senecionine *N*-oxide (**4**), seneciphylline (**5**), retrorsine (**6**), integerrimine (**7**), lasiocarpine (**8**) and heliotrine (**9**), were analysed using this technique.

EXPERIMENTAL

Chemicals. Acetonitrile and ammonium acetate were HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA). Nanopure water was prepared by filtering distilled water through a 45 μm filter. The pyrrolizidine alkaloids were available from the laboratory of a co-author (R.J.M.).

Plant material and extracts. The extracts of *Senecio spartioides*, *S. douglasii var. longilobus*, *S. jacobaea* and *S. intergerrimus var. exaltatus* were available from the laboratory of a co-author (R.J.M.). Roots of *Symphytum officinale* were purchased from Frontier Natural Products (Frontier, IA, USA).

Preparation of extracts. The extraction procedure reported by Molyneux and Johnson (1984) was followed. A weighed amount of plant material was extracted overnight with methanol. After filtration, the filtrate was concentrated and the extract partitioned between

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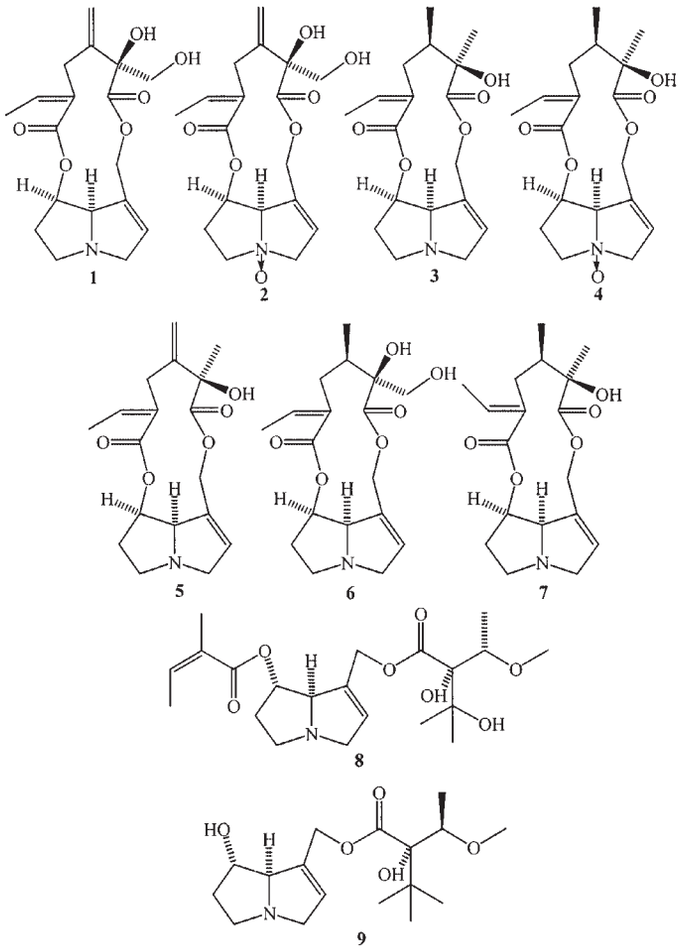

chloroform and 2 M hydrochloric acid. The two phases were separated and the aqueous phase was stirred overnight with zinc dust and then basified. The basic aqueous fraction was extracted with chloroform, and the chloroform removed to provide a pyrrolizidine alkaloid extract for analysis. An aliquot (ca. 10–12 mg) of extract was placed in a 5 mL volumetric flask and dissolved in methanol. Samples were filtered through 45 μm nylon filters (Phenomenex, Torrance, CA, USA) prior to injection.

HPLC-ELSD analysis. A Waters (Milford, MA, USA) module I liquid chromatograph was interfaced with a UV detector and a Sedex (SEDERE, Alfortuille, France) model 55 ELSD operated at 40°C and 2.3 bar nitrogen. A Waters XTerra RP_{18} column (150 × 4.6 mm; 5 μm particle size) was used at ambient temperature. The mobile phase consisted of water containing 0.015 M ammonium acetate (solvent A) and acetonitrile (solvent B). At a flow rate of 0.75 mL/min, the gradient elution (A:B) was from 95:5 to 50:50 in 40 min. After injection of 10 μL of sample, data was collected and analysed using Waters Millennium[32] software. The limit of detection was determined to be 40 μg/mL.

RESULTS AND DISCUSSION

HPLC with UV detection is the most common method of analysis of pyrrolizidine alkaloids. There are, however, limitations when using a UV detector, in particular those pyrrolizidine alkaloids not having a chromophore in their structure are weakly detected, at best, even at short wavelengths. This is demonstrated in Fig. 1. A mixture of nine pyrrolizidine alkaloids was analysed by HPLC and chromatograms detected at 210 and 254 nm and also by ELSD. The peak corresponding to **9** (heliotrine) was at or below the detection limit when measured by UV due to its lack of chromophore. Therefore, a sensitive detection by UV could not be achieved, whereas the chromatogram obtained by ELSD showed a clear detection of **9** comparable to the pyrrolizidine alkaloids containing a chromophore.

The utility of ELSD was demonstrated by analysing plant extracts known to contain hepatotoxic pyrrolizidine alkaloids, namely, *Senecio spartioides*, *S. douglasii var. longilobus*, *S. jacobaea*, *S. intergerrimus var. exaltatus* and *Symphytum officinale*. At least one pyrrolizidine alkaloid was detected in each extract (Table 1). Lasiocarpine (**8**) was detected in *S. officinale. Senecio spartioides* and *S. douglasii var. longilobus* both contained compounds **1**, **3**, **5** and **6** but, as shown in Fig. 2, the quantity of **1** and **5** varied greatly between the two. The constituents **3**, **5** and **6** were detected in *S. jacobaea* but only compound **1** was detected in *S. intergerrimus var. exaltatus*. Since each *Senecio* species had a distinctly different chromatogram, it is possible that a chemical profile could be developed in order to distinguish between *Senecio* species. This may be beneficial when an adulteration in a food source for humans or livestock is detected.

Hosch *et al.* (1996) demonstrated that the choice of extraction procedure has an effect on which form of pyrrolizidine alkaloid, i.e. *N*-oxide or free-base, is

Table 1. Pyrrolizidine alkaloids detected by ELSD in various plant extracts

	Alkaloids						
Plant	**1**	**3**	**5**	**6**	**7**	**8**	**9**
Senecio spartioides	+	+	+	+	–	–	–
S. douglasii var. longilobus	+	+	+	+	–	–	–
S. jacobaea	–	+	+	+	–	–	–
S. intergerrimus var. exaltatus	+	–	–	–	–	–	–
Symphytum officinale	–	–	–	–	–	+	–

1, Riddelliine; **3**, senecionine; **5**, seneciphylline; **6**, retrorsine; **7**, integerrimine; **8**, lasiocarpine; **9**, heliotrine. +, detected; – not detected.

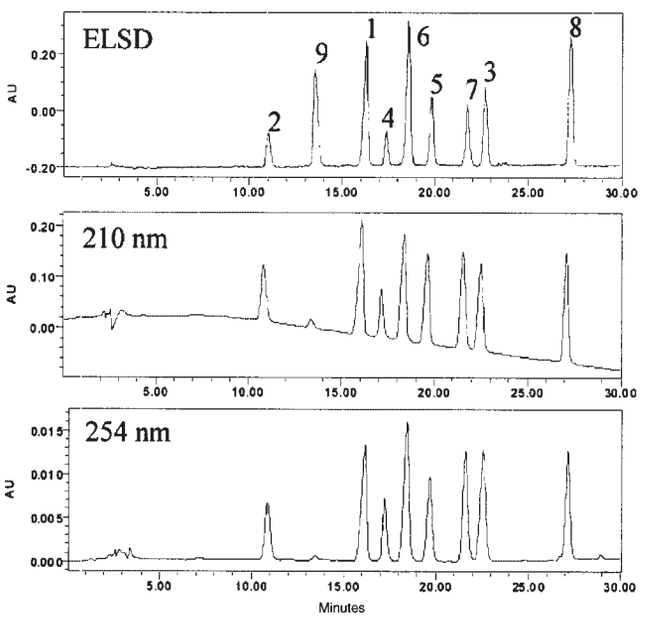

Figure 1. HPLC chromatograms of a mixture of pyrrolizidine alkaloids detected by ELSD, and by UV detection at 210 nm and 254 nm. Key to peak identities: **1**, riddelliine; **2**, riddelliine *N*-oxide; **3**, senecionine; **4**, senecionine *N*-oxide; **5**, seneciphylline; **6**, retrorsine; **7**, integerrimine; **8**, lasiocarpine; and **9**, heliotrine. (For chromatographic protocol see Experimental section.)

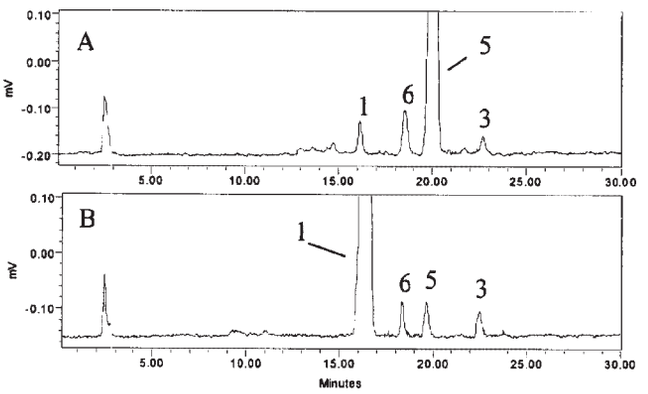

Figure 2. HPLC chromatograms of extracts of (A) *Senecio spartioides* and (B) *Senecio douglasii var. longilobus* detected by ELSD. Key to peak identities; **1**, riddelliine; **3**, senecionine; **5**, seneciphylline; and **6**, retrorsine. (For extraction and chromatographic protocols see Experimental section.)

predominantly extracted. According to this study, extraction at room temperature with methanol yielded a ratio of *N*-oxide to free-base of ca. 85:15. On the other hand, when the extraction procedure involved heating (i.e. as in Soxhlet extraction), the ratio varied depending on the extraction time (being 80:20 after 4 h extraction and 40:60 after 21 h extraction). The alkaloids were also selectively extracted by submitting a methanol extract to solid phase extraction (SPE) (Hosch *et al.*, 1996) following which both *N*-oxides and free-bases were present in the chromatograms. In the present study, the *N*-oxides were reduced to their free-base form by reduction of the extract and hence no *N*-oxides were detected in the chromatograms.

Although the limits of detection may be lower by photodiode array detection (ca. 1 μg compared with ca. 40 μg by ELSD) or by mass spectrometric detection, the cost of purchase and maintenance of these types of equipment is much greater. As shown, ELSD is an important addition for the analytical detection of hepatotoxic pyrrolizidine alkaloids in plant extracts: owing to its low cost and low maintenance requirements, its utility can be more wide spread than that of mass spectrometric detection.

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